Review

Nanovaccines: recent developments in vaccination

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In the past 100 years, vaccination has contributed immensely to public health by preventing a number of infectious diseases. Attenuated, killed or part of the microorganism is employed to stimulate the immune system against it. Progress in biotechnology has provided protective immunity through DNA vaccines. In recent years, nanovaccine is a novel approach to the methodology of vaccination. Nanomaterials are delivered in the form of microspheres, nano-beads or micro-nanoprojections. Painless, effective and safe needle-free routes such as the intranasal or the oral route, or patches of microprojections to the skin are some of the approaches which are in the experimental stage at present but may have a great future ahead in nanovaccination.

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1. Introduction

By definition, immunization is the deliberate stimulation of an adaptive immune response. Immunization, also called vaccination or inoculation, is a method of stimulating resistance in the human body to specific diseases using microorganisms – bacteria or viruses – that have been modified or killed. These treated microorganisms do not cause diseases, but rather trigger the body's immune system to build a defence mechanism that continuously guards against the disease. If a person immunized against a particular disease later comes in contact with the disease-causing agent, the immune system is immediately able to respond defensively.

Worldwide vaccination programmes have eradicated diseases such as smallpox, diphtheria, poliomyelitis and neonatal tetanus in most of the developed and some developing countries. Thus, immunization has reduced the incidence of such deadly diseases.

Genetic engineering techniques have been used to further improve the strategy for vaccination by isolating a gene or genes within an infectious organism that codes for a particular antigen. Subunit vaccines for hepatitis B and pneumococcal infection, which causes pneumonia, are safe to use as they do not cause the disease.

2. DNA vaccines

A vaccination strategy currently under development for a number of diseases utilizes plasmid (circular piece of bacterial) DNA that encodes antigenic proteins. This is injected directly into the muscle of the recipient. Muscle cells take up the DNA, and the encoded protein antigen is expressed, leading to both humoral and cell-mediated immune responses. Local dendritic cells (DCs) may play an important role in the development of antigenic responses to DNA vaccines. As only a single microbial gene or DNA-encoding set of antigenic peptides is used, it is safe and

Keywords. Nanotechnology; DNA vaccines; immunization; nano-beads

Abbreviations used: BSA, bovine serum albumin; DC, dendritic cell; EPA, Environmental Protection Agency; FMDV, foot and mouth disease viruses; HbsAg, hepatitis B surface antigen; HPV, human papillomavirus; ISCOM, immune-stimulating compound; MHC, major histocompatibility complex; mNP, magnetic nanoparticles; paNP, polyacrolein nanoparticles; PLG, polylactide-co-glycolide; PLGA, polylactide-co-glycolide acid; siRNA, small interfering RNA; USFDA, US Food and Drug Administration; VLP, virus-like particle; W/OW, water-in-oil-in water

does not carry the risk of active infection. Furthermore, DNA vaccines offer advantages over many of the existing vaccines, e.g. the encoded protein is expressed in the host in its natural form – there is no denaturation or modification, and they cause prolonged expression of the antigen, which generates significant immunological memory (Janeway *et al.* 2005).

3. Veterinary vaccines

The requirements for veterinary vaccines are different for different animals and also from those for human vaccines. Although more side-effects can be tolerated by animals than by humans, as public pressure on animal welfare increases, it is likely that less reactogenic vaccine preparations will be favoured. Another aspect of veterinary vaccines is the genetic diversity of the species. The diseases in different species vary and therefore vaccine requirements will differ among species. In wildlife species, knowledge of targeting molecules (cell surface markers and immune modulators) is minimal, which is a limiting factor. Environmental issues dominate in the use of vaccines for wildlife. On the other hand, the regulatory requirements for biodegradables could be much stricter for animals bred for human consumption than those used as pets (Scheerlinck and Greenwood 2006). The route of delivery can vary from oral administration in pets to long-distance ballistic intramuscular delivery in wild animals. The size of the particles can range from a few nanometers to 1 cm: biobullets (1–2 cm) shot with an air rifle at wild animals, microparticles (1–100 μm) for DNA vaccination and nano-beads (10–500 nm) as adjuvants in injected formulations.

4. Cancer vaccines

The scenario for human vaccines is different, especially for cancer patients. Chemotherapy, radiotherapy and surgery are the three major treatments available. In recent years, scientists and clinicians have learnt more about how the body fights cancer on its own. This has helped in developing therapies to prevent some forms of cancer. Targeted therapy is one of the most attractive areas in the treatment of cancer due to the broad array of therapeutic options available for the approach, including potent immune cytotoxic activity, growth factor receptor inhibition, and potential selective delivery of toxins, isotopes and drugs to tumours. Cancer vaccine is another therapy which is emerging, although it is in the experimental stage. Two vaccines that can help to prevent cancer have been approved by the US Food and Drug Administration (FDA). One of these vaccines prevents infection with the human papillomavirus (HPV), which causes cervical cancer (*www.en.wikipedia.org*). Drugs can be specifically formulated to attack tumour cells and destroy them without affecting the normal cells. This will be an ideal situation for the treatment of cancers. Recent advances in the formulation of nanoparticles and nanostructures for targeted therapy emerge as a challenge in designing and delivering these drugs.

5. Nanotechnology

Nanotechnology is the development of engineered devices at the micromolecular level in the nanometer range. One nanometer is one billionth of a meter (a DNA strand is 2 nm wide) and is the width of about five atoms (Navalakhe and Nandedkar 2007). Due to their small size and large surface area which enhances their action, nanomaterials and nanodevices are being developed for early diagnosis of cancer and infectious diseases. Nanomedicine is a cutting-edge area of research that combines the concepts of nanotechnology and medicine, and provides new hope for research in this field (Seetharam 2006). The idea that a nanostructure could be designed, manufactured and introduced into the human body to improve health, including cellular repairs at the molecular level, is encouraging (Freitas 2005). The nanomaterial is so small that it can easily enter the cell; therefore, nanomaterials can be used *in vivo* or *in vitro* for biological applications. This has led to the development of diagnostic devices, contrast agents, analytical tools, physical therapy applications and drug delivery vehicles. Advances in nanotechnology have also proved to be beneficial in therapeutic fields such as drug discovery, drug delivery and gene/protein delivery. Drug consumption and side-effects can be significantly lowered by depositing the active agent at the desired location. This concept has been found to be useful in developing nanovaccines using different routes of administration.

5.1 *Oral route*

In gene therapy, local or systemic administration of a nucleic acid construct can prevent, treat or cure disease by altering the expression of gene(s) that are responsible for the pathological condition. Oral gene therapy is promising for diseases such as inflammatory bowel disease.

Further, efficient oral delivery of DNA vaccines is possible. The development of polymer-based nanoparticles is useful in the delivery of oral DNA vaccines (Bhavsar and Aniji 2007). Further extensive study in this area could reveal the possibility of using oral DNA vaccination against diseases. Due to dilution during the transport of the vaccine through the gastrointestinal tract, a higher concentration is required for the vaccine to be effective. This can be a drawback of the oral route of administration.

5.2 *Nasal route*

Needle-free nasal immunization, using nanoemulsion with hepatitis B antigen, has been reported to be a safe and effective hepatitis B vaccine (Makidon *et al.* 2008). This nanoemulsion is made of soya bean oil, alcohol, water and detergents emulsified into droplets of 40 nm. This vaccine does not require refrigeration and is stable for 6 months. Further, 2 shots of the vaccine can be given instead of 3 shots of the conventional vaccine for hepatitis B in animals. Alum, used as an adjuvant in the conventional vaccine, causes irritation, pain and redness. The new nanoemulsion is non-toxic, pain free and avoids the risk of spreading needle-borne infections. Baker's group (Makidon *et al.* 2008) affirms the promise of a nasal nanoemulsion strategy for smallpox, influenza, anthrax and HIV vaccines. Results obtained by his group in mice, rats and guineapigs showed that three types of immunity – systemic, mucosal and cellular – could be achieved. Toxicological studies have shown that the vaccine is safe and well tolerated. Current research on hepatitis B vaccines has also elicited protective antibodies in the blood of animals with the nanoemulsion approach as an effective adjuvant.

Baker's group further reported a strong response against whole virus in smallpox vaccine. This nanoemulsion vaccine was against killed viruses and offered equal protection as compared with the existing one and further avoided the use of inflammatory adjuvant, e.g. hydroxide. They also conducted preliminary testing of a nanoemulsion against GP 120, one of the major binding proteins, and reported that this vaccine could induce mucosal and cellular immunity, and neutralize antibody to various isolates of HIV. These exploratory studies may have implications for viral vaccines.

Intranasal delivery of vaccines poses two major challenges: the first is accurate and repeated dispensing of very small quantities of formulated vaccine and the second is deposition of the formulated vaccine to all areas of the nasal mucosa (especially lymphoid tissues) while limiting the deposition of particles in the lung (Sharma *et al.* 2009). Nasal drugs are manually delivered by pipette or spray pump but these devices have many disadvantages such as local irritation, inadequate distribution of particles/droplets and unpleasant taste from concentrated drug reaching the mouth. At present, unit or duo-dose nasal spray devices are used for the delivery of vaccines. These are small, disposable devices that deliver a limited number of sprays which are 'easy to use and ready to use'. Flumist for influenza vaccine is an example that has been marketed (AIDS R & D Profile 2003). Another novel nasal spray device is the bidirectional nasal delivery device that takes advantage of the posterior connection between the nasal passage, which persists when the soft palate automatically closes during oral exhalation. Thus, the release of liquid or particles into the airflow enters one nostril via a sealing nozzle and exits through the other nostril. The bidirectional nasal delivery concept minimizes the risk and problems related to deposition of particles in the lung, which occurs during conventional inhalation from a nebulizer, and increases the delivery of particles to the posterior part of the nasal mucosa. The use of bidirectional nasal delivery devices for nasal administration of antigen such as influenza and diphtheria are under clinical trials and limited studies have shown promising results (Djupesland *et al.* 2006).

The main hurdle in the nasal administration of antigens is their delivery, as free antigens are readily cleared from the nasal cavity, poorly absorbed by the nasal epithelial cells and generate a low immune response. To overcome these problems, encapsulation of the antigen into bioactive nanoparticles is a promising approach to nasal vaccine delivery (Slutter *et al*. 2008).

As it is needle-free, the nasal route offers significant benefits over the other routes of administration for vaccine delivery, but its direct access to the brain and potential problems with allergy and respiratory syndromes such as asthma require further studies to ensure the safety of the intranasal route of immunization for human application.

5.3 *Intradermal route*

It is challenging to deliver drugs and genes through the tough layer covering the skin into the underlying epidermal cells which are immunologically sensitive. For vaccination, a needle attached to a syringe penetrates the outer layer of the skin and reaches the epidermal cells to deliver the drug from the syringe. The drug emulsified with an adjuvant forms a depot so as to allow slow release of the drug over a long duration.

Three kinds of adjuvants are frequently used, particulate ones such as oil emulsions, non-particulate such as saponins and combined compositions, e.g. immunostimulating complexes. Adjuvants help in slow release of the antigen, targeting antigens to the relevant antigen-presenting cells of the immune system or directly activating the cells of the immune system. Sinyakov *et al.* (2006) used biodegradable magnetic nanoparticles (mNP, 100–150 nm) and monodispersed polyacrolein nanoparticles (paNP, ~200 nm), inert submicron polymeric particles as adjuvants, which are covalently bound to bovine serum albumin (BSA). They compared the immune response in mice with conventional adjuvants – incomplete Freunds and alum (aluminium potassium sulphate). Anti-BSA antibody response with BSA–paNP was superior to BSA–mNP and was comparable to that of BSA alum.

6. Polymer-based vaccination

Proteins and peptides, either purified from microorganisms or tissues, synthesized chemically or produced by recombinant

DNA technology or expressed by the relevant DNA construct, are often weakly antigenic. To be used as effective vaccines, these proteins and peptides require immune-stimulating compounds (ISCOMs) or adjuvants which act non-specifically to increase the immune response of these weak antigens. Some of the currently used conventional vaccines are inefficient as they lack appropriate adjuvants. Therefore, harmless and effective adjuvants are needed in modern vaccinology. In recent years, various approaches have been tested, one of these being the development of nanoparticles prepared from biodegradable and biocompatible polymers as a vaccine delivery system to induce both humoral and cellular immune responses.

Use of a particulate adjuvant for vaccine delivery protects the antigen from degradation and impact during penetration through the skin and muscle. Further, particulate vaccine delivery systems can induce cytotoxic T lymphocyte (CTL)- and other cell-mediated immunity to key pathogens including viral infections. DNA delivery can be achieved by gold-coated particles, liposomes, inert macro-, microspheres and nano-beads. Inert particles coated or conjugated to antigen are designed to provide a targeted immune response. Encapsulation of antigen has been widely used for its easy delivery, slow release and protection from degradation of the antigen with an effective single dose. Nanoparticles range from 10 to 500 nm, while microparticles are larger, around 1–100 μm in diameter. Solid particles are made from inert materials or biodegradable polymers. Biodegradable and biocompatible polyesters such as polylactide-co-glycolides (PLG) have been extensively used to encapsulate antigens. After hydrolysis of α-hydroxyl acids, PLG forms lactic and glycolic acids, yielding small spherical polymeric particles 1–100 nm in size. The encapsulation process involves exposure of the antigen to organic solvents. High shear stress due to the low pH during the degradation of the polymer may compromise the integrity of the encapsulated antigen. However, PLG microspheres have been used successfully for intranasal immunization incorporating *Toxoplasma gonadii* in sheep (Stanley *et al.* 2004) *Staphylococcus aureus* in cows (O'Brien *et al.* 2000) and *Pasteurella multocida* antigen with cholera toxin in rabbits (Suckow *et al*. 1996). Water-dispersed liquid nanoparticles of 10–500 nm have been tried. Immune response has been demonstrated against atrophic rhinitis or pleuropneumonia with liquid nanoparticles combined with ISCOMs in swine with no adverse reactions (Aucouturier *et al*. 2001). Thus, nano- and microparticles have the ability to control the release of encapsulated antigen and are safe, suggesting that they may be applied for veterinary vaccines.

Nanoparticles prepared from biodegradable polymer polylactide-co-glycolide acid (PLGA) have been used for encapsulating peptide (reviewed by Bharati *et al.* 2007). Chitosan-modified PLGA microspheres produced both humoral and cellular immune responses when a single dose was administered intranasally in rabbits. DNA-based vaccines can provide long-lasting immunity. However, promising DNA vaccines may not be as active in humans as in animals. To increase the efficacy of DNA-based vaccines, DNA encoding hepatitis B surface antigen (HBsAg)-encapsulated formulation of PLGA nanoparticles could induce enhanced immunity in mice (He *et al.* 2005). Recently, our attempt to formulate nanoparticles of octapeptide using PLGA has revealed that the bio- and immunoactivity of the peptide was retained (Patel *et al.* 2008). Yet, no successful clinical trials with the PLGA-encapsulated nanoparticle antigen have being reported, suggesting that extensive experimental work is needed in this promising area.

7. ISCOM-based adjuvants

Hydrophobic membrane-associated viral antigens have been incorporated in 40 nm particles of immune-stimulatory complexes as adjuvants for initiating an immune response. The majority of applications for ISCOM-based adjuvants in veterinary species are for vaccines against viral diseases (reviewed by Morein *et al.* 2004). Vaccine against gonadotrophin-releasing factor using ISCOM-based adjuvant has been marketed as Equity (Pfizer Animal Health), which acts as an anticonception vaccine for horse.

8. Virus-like particles (VLPs)

Advances in recombinant systems such as Baculovirus led to the expression of large quantities of viral proteins. With the availability of the three-dimensional structures of these viruses, it is possible to engineer vaccines with multiple viral epitopes in order to elicit protective immunity (Roy and Sutton 1998). VLPs co-administered with oil, mutant *E. coli* heat-labile toxin in gnotobiotic calves (Han *et al*. 2006) and with blue tongue virus in sheep (Roy 2003) have provided protection against the respective viruses. The immunogenicity of VLPs is possibly due to their interaction with DCs and has been a useful approach for immunity against viruses in animals.

9. Nano-beads

Particulate immunogenic carriers such as ISCOMs and VLPs, as well as most of the common viruses are 20–200 nm in diameter. Yet, for most of these vaccines adjuvants are essential for an appropriate immune response, while particulate vaccines appear to induce immune responses without adjuvants. Solid inert beads with surface-adsorbed antigen have been shown to stimulate CD8 T cell responses (Scheerlinck *et al.* 2006). The antigen conjugated to the

bead (figure 1) and the size of the bead play a major role in eliciting a combined response of humoral and cell-mediated immunity. Fifis (2004) reported that a covalently conjugated antigen-bead preparation promoted higher T-cell responses than antigen simply mixed with nano-beads or soluble antigen without the bead carrier. The 40 nm beads are preferentially localized to the DCs in the draining lymph nodes, and thereby activate DCs to induce a cell-mediated immune response. Nano-beads were consistently the best adjuvant for induction of CD8 T cell-mediated responses with whole antigen (ovalbumin). Antigen conjugated to nano-beads was therefore very effective in stimulating both specific antibodies and major histocompatibility complex (MHC) class I-restricted T cell immunity (Fifis *et al.* 2004). Antigen covalently linked to inert nano-beads with a size of ~50 nm is preferentially taken up by DCs, thus inducing humoral as well as cell-mediated immune responses (Fifis *et al.* 2004 and Scheerlinck *et al.* 2006). Fifis and colleagues have further reported therapeutic and protective properties of nanovaccines against tumours. They covalently conjugated antigen to solid core beads of 40 nm that localized to DCs in the draining lymph nodes, inducing high levels of interferon-γ production and high antibody titres in mice. These responses were higher than those with conventionally used adjuvants such as alum. A single dose of antigen-conjugated beads protected mice from tumours and caused rapid clearance of established tumours in two different mice models. Thus, nano-beads are effective as immunogens, for therapeutic as well as preventive purposes.

A major problem observed with current vaccines is that some of the vaccinations, e.g. foot and mouth disease viruses (FMDV) do not allow differentiation between infected and vaccinated animals. Greenwood *et al.* (2008) recently reported that a combination of multiple peptides, either conjugated separately to individual nano-beads or to the mixture, induces significant cell-mediated and humoral immune responses compared with single-peptide conjugations in sheep. For the generation of vaccines against a range of genotypes, a combination of peptides conjugated to nano-beads may be the best solution as they induce both B and T cell responses. Thus, combining several peptides in the peptide–nano-bead based vaccine approach can improve immunogenicity and may prove beneficial, especially for highly variable pathogens such as FMDV (Greenwood *et al*. 2008).

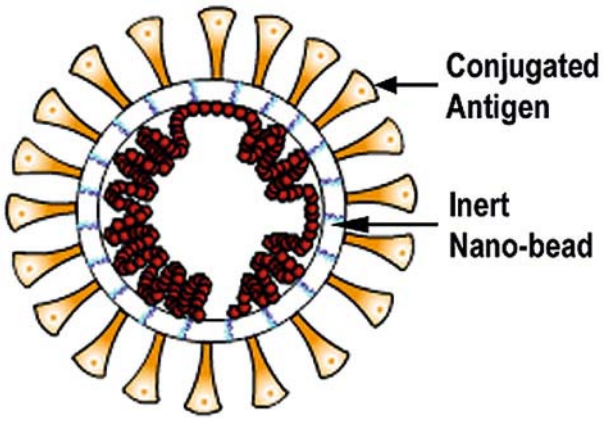

Figure 1. Solid inert bead with surface-adsorbed antigen

10. Micro-needle arrays

Various technologies have been employed for delivering drugs – needle–syringe, liquid jet injectors, micro needle arrays/patches and biolistic particle injection (Kendall 2006). The advantage of a needle-free system is that it is painless and more efficient in delivering drug, plasmid DNA and protein. It is a practical alternative to the needle–syringe route for targeted delivery of vaccines.

Skin has an outermost layer called the stratum comeum, which is 10–20 μm thick, below which lies the viable epidermis that extends up to 50–100 μm in depth. The latter comprises 2% of Langerhans cells (10 μm) evenly distributed in this layer. These are extremely effective antigen-presenting cells, responsible for the uptake and processing of foreign materials to which they generate an immune response. These cells are 1000 times more effective than keratinocytes, fibroblasts and myoblasts, causing immune response to a variety of polynucleotides or antigens (Chen *et al.* 2002). This property is advantageous in developing vaccinations against viruses causing diseases, such as HIV and cancer.

The most common method for the delivery of vaccination is by the use of a small-gauge needle attached to a syringe. Injection into the skin, although beneath the stratum corneum, may not practically reach the target, i.e. Langerhans cells of the viable epidermis. Therefore, vaccine is administered intramuscularly, but with DNA, the immune response is modest due to indirect targeting of the DCs. In addition to this, a needle prick can be painful and, at times, irritating. In view of this, needle-free methods are being developed in recent years. These include diffusion patches, liquid jet injections and micro-needle arrays. Each of these has its advantages as well as drawbacks.

Diffusion through patches applied to skin is one of the least invasive methods for the delivery of small molecules (<500 Da). The transport of large molecules has also been attempted using simple approaches such as tape stripping with an adhesive tape and brushing with sandpaper. In addition, electroporation, ablation by laser or heat, radiofrequency high-voltage currents, ionophoresis, liposomes, sonophoresis and microporation are some of the

advanced technologies being explored, but have yet to be tested for vaccines and immunotherapies (Kendall 2006). Liquid jet injector is an approach in which DNA vaccine in liquid is delivered by a high-speed injector around the Langerhans cells. However, it disrupts the epidermal and dermal layers of the skin and therefore needs controlled delivery, which is being tested.

Skin is a sensitive immune organ containing a network of antigen-presenting cells and cells with innate and adaptive immune functions. In addition to this, skin is an easily accessible and practical site for administration of vaccination. Taking advantage of these characteristics, a few needle-free technologies have emerged since the past 5 years. Skin delivery technologies provide potentially safer alternatives to needle injections and promise increased efficacy for the prevention and/or therapy of infectious diseases, allergic disorders and cancer. Chen (2002) reported that epidermal powder immunization and particle-mediated gene-gun DNA immunization use similar mechanical devices to deliver protein and DNA vaccines, respectively, into viable epidermis. A contoured shock-tube concept and its embodiment device were employed by Liu and Kendall (2007) to provide a controllable system for transdermal delivery of microparticles. Kendall's group (Chen *et al.* 2008) has explored the novel approach of using arrays of micro nanoprojections on patches to achieve a physical targeted method of needle-free delivery of vaccines to skin. Conceptually, the delivery device is a set of needles of microscale length with their nanoscale tips coated with the drug that is applied to the skin as a small patch. The patch is painless and needle-free and can accurately, efficiently and safely deliver biomolecules to the viable cells of the epidermis. Compounds such as ethidium bromide, plasmid DNA and proteins can be uniformly and controllably coated onto very small and densely packed micro nanoprojections using a single, versatile technique. Scanning electron microscope images of micro nanoprojection patches, before and after coating with plasmid DNA, revealed that a uniform DNA coating could be achieved on these small and densely packed projections (figure 2). By confocal microscopy, the group further found that, following the application of coated patches on mouse ear skin for a few minutes, the coating was able to pierce the skin and deliver drug, protein and plasmid DNA successfully into the epidermis. These novel experiments indicate that vaccination can be needle-free, and yet more efficient and safe in the near future and can be used as therapy for curing diseases.

Recently, Tran *et al.* (2008) developed a unique nanoliposomal ultrasound-mediated device for delivering small interfering RNA (siRNA) specifically targeting ^{V600E}B-Raf and Akt3 into melanocytic tumours present in the skin. They observed that the topical delivery of cationic nanoliposomes loaded with siRNA in combination with low-frequency ultrasound has the potential to decrease early melanocytic lesion development in the skin and prevent the spread of cutaneous metastases of melanoma.

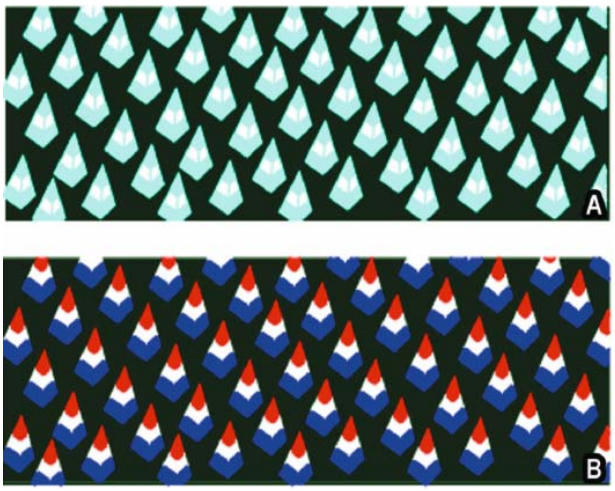

Figure 2. Diagrammatic representation of scanning electron microscopy images of **(A)** unloaded and **(B)** plasmid DNA-coated micro-nanoprojection patches.

11. Nanovaccines: advantages and disadvantages

Microparticles covalently coupled with antigen offer distinct advantages – a low dose of antigen is required, efficient processing by antigen-presenting cells and stability during storage (Gengoux and Leclerc 1995).

Encapsulation of antigen has been widely used as it is easy to deliver, protects the antigen from degradation and is found to be effective with a single dose due to slow release of the antigen. The use of microparticles thus improves immunogenicity due to the absence of adjuvants such as alum, which are inflammatory mediators.

Alum, a common adjuvant used in conventional hepatitis B vaccine, is known to cause irritation. In contrast, the use of needle-free nasal immunization with a combination of nanoemulsion and hepatitis B antigen was found to be tolerable and effective. It was also safe as no inflammation and no evidence of the vaccine in the olfactory bulb was observed by Makidon *et al.* (2008). Interestingly, refrigeration was not required for this nanoemulsion, as it was effective for a month at 25°C, and for 6 weeks at 40°C, thereby facilitating its final distribution in small areas/villages of developing countries.

Despite the availability of a number of vaccines in the market for prevention of diseases, their cost per dose and delivery of multiple doses are the limiting factors of these vaccines. In addition to this, the requirement for cold storage is another disadvantage of conventional vaccines. In view of this, efforts have been made to develop nano/microparticles

prepared from biodegradable and biocompatible polymers as vaccine delivery systems to induce both humoral and cellular immune responses. Significant advantages of these biodegradable polymers are their long history of safety, proven biocompatibility, and their property to control the time and rate of polymer degradation and antigen release (Kersten and Hirshberg 2004).

The most commonly used method for the preparation of antigen-encapsulated nano/microparticles is the solvent extraction or evaporation from a water-in-oil-in water (W/O/W) emulsion (Patel *et al.* 2008). A primary emulsion (W/O) is formed by homogenizing or sonicating an aqueous solution of the antigen with a polymer in organic solvent. The ratio between the aqueous phases is variable and is an important factor. The major problems encountered with this technology are reproducibility of the production of soluble nanosize (40 nm) formulation, aggregation and detection of immune responses. These problems therefore need to be addressed, with follow up using clinical studies (Kalkanidis *et al.* 2006).

Entrapment efficiency, release kinetics and other physical characteristics such as morphology, porosity and size distribution, which influence the efficacy of the formulation, can be controlled by using an appropriate combination of different polymers. This can lead to successful formulation of a vaccine.

Many of the nanovaccines are non-invasive, delivered by the oral or nasal route, diffusion patches or microneedle arrays, thus allowing pain-free delivery with minimal damage. This is an advantage over conventional vaccines, which are usually multi-injection, multi-dose delivery systems (Kendall 2006).

Regulatory policies are based mainly on the purity and safety of the vaccine. In addition to this, stability during production and storage, sterilization by non-thermal methods such as gamma radiation, reproducibility of formulation during industrial manufacturing are some of the hurdles that need to be resolved.

Reproducibility of formulation during manufacturing is one of the major hurdles in the use of nanoparticles as vaccines (Sharma *et al.* 2009), as size-dependent immunogenicity has been reported by Fifis *et al.* (2004). Chen's group (Zhang *et al*. 2006) has also observed that nanomaterials can change size, shape but not composition, which may change their toxicity.

Although small nanoparticles are cleared quickly from the body, large counterparts may accumulate in vital organs causing toxic problems. In view of this, toxicologists have created batteries of high-speed assays for testing the toxicity of nanomaterials with a hope that these throughput assays could speed up toxicological screening (Service 2008). However, with nanovaccines, the particles are cleared slowly over a prolonged time period, and this may induce toxic effects. Therefore, evaluation of the safety of nanovaccines is equally essential as the study of their efficacy (Nandedkar *et al.* 2008).

12. Nanotoxicity

The traditional toxicology studies that test the required material appear to be inadequate and hence hamper the progress of application of nanoparticles (Service 2008). Nel *et al.* (2006) from the University of California, LA, USA and other toxicologists reported batteries of high-speed assays for testing the toxicity of hundreds and thousands of nanomaterials at the same time. In these 'assays', different types of cells are exposed to nanoparticles and their effect on DNA damage, gene expression, cell death and growth are evaluated. These high-throughput studies can at least prioritize nanomaterials that should undergo conventional toxicology tests.

Unfortunately, *in vivo* studies are expensive. A single *in vivo* animal study can cost US$ 50 000. Further, it is at times difficult to extrapolate the results obtained from one tissue to another. *In vitro* assays in cultured cells, although unlikely to substitute for animal studies, could help dissect structure–activity relationships. The Environmental Protection Agency (EPA) in Research Triangle Park, NC, USA in 2007 launched 'ToxCast', a wide variety of cell-based assays to test chemicals looking for 400 different end-points that could signal danger to testing organisms. Houck, an EPA toxicologist, is helping in the development of this program. In recent years, the number of consumer products containing nanoparticles has nearly doubled, and these require testing for toxicology. In a recent study, Weissleder at Harvard Medical School, Boston evaluated 50 different nanomaterials at four concentrations on four cell types with four different assays. Shaw and colleagues (2008) found broad, consistent patterns of activity and a heterogeneous range of responses. Although a single test cannot predict the toxicity of nanomaterials, batteries of cell assays can help decide the likely safety in human studies. Thus, the efforts of Nel, Chen, Houck and Weissleder will help to speed up toxicological screening of nanomaterials by using high-throughput assays and accelerate the application of nanomaterials for clinical trials to prevent/cure diseases in the near future.

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